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1: Iwaki K, Ogawa M, Tanaka S, Kosaki G.
Radioimmunoassay for human pancreatic chymotrypsin and measurement of serum immunoreactive chymotrypsin contents in various diseases.
Res Commun Chem Pathol Pharmacol. 1983 Jun;40(3):489-96.
PMID: 6622821 [PubMed - indexed for MEDLINE]

2: Largman C, Brodrick JW, Geokas MC.
Radioimmunoassay determination of circulating pancreatic endopeptidases.
Methods Enzymol. 1981;74 Pt C:272-90. No abstract available.
PMID: 7033725 [PubMed - indexed for MEDLINE]

3: Geokas MC, Largman C, Brodrick JW, Johnson JH, Fassett M.
Immunoreactive forms of human pancreatic chymotrypsin in normal plasma.
J Biol Chem. 1979 Apr 25;254(8):2775-81. No abstract available.
PMID: 429317 [PubMed - indexed for MEDLINE]

Thank you.
Happy New Year!

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forms,[57] its role in various metabolic processes,[44,58,59] and its biosynthesis.[60–63]

The specificity of these antibodies, which should be enhanced by recent developments in monoclonal antibody technology, promises that their use in the study of these proteins will continue to grow in future years.

[57] P. E. Thomas, D. Korzeniowski, D. Ryan, and W. Levin, *Arch. Biochem. Biophys.* 192, 524 (1979).
[58] P. E. Thomas, A. Y. H. Lu, S. B. West, D. Ryan, G. T. Miwa, and W. Levin, *Mol. Pharmacol.* 13, 819 (1977).
[59] G. T. Miwa, W. Levin, P. E. Thomas, and A. Y. H. Lu, *Arch. Biochem. Biophys.* 187, 464 (1978).
[60] R. N. DuBois and M. R. Waterman, *Biochem. Biophys. Res. Commun.* 90, 150 (1979).
[61] M. B. Cooper, J. A. Craft, M. R. Estall, and B. R. Rabin, *Biochem. Biophys. Res. Commun.* 91, 95 (1979).
[62] R. A. Colbert, E. Bresnick, W. Levin, D. Ryan, and P. E. Thomas, *Biochem. Biophys. Res. Commun.* 91, 886 (1979).
[63] S. Bar-Nun, G. Kreibach, M. Adesnik, L. Alterman, M. Negishi, and D. D. Sabatini, *Proc. Natl. Acad. Sci. U.S.A.* 77, 9650 (1980).

[17] Radioimmunoassay Determination of Circulating Pancreatic Endopeptidases

By COREY LARGMAN, JAMES W. BRODRICK, and MICHAEL C. GEOKAS

Introduction

In the past several years, the radioimmunoassay technique has been applied to the detection of pancreatic endopeptidases in plasma and serum. These studies have demonstrated that while the bulk of the digestive enzymes enters the pancreatic ductal system, a small fraction enters the bloodstream. The precise mechanism of this exocrine–endocrine partition of pancreatic enzymes is not well understood. The application of the radioimmunoassay methodology has permitted the characterization of the circulating molecular forms of human cationic trypsin,[1,2] anionic trypsin,[3]

[1] M. C. Geokas, C. Largman, J. W. Brodrick, and J. H. Johnson, *Am. J. Physiol.* 236, E77 (1979).
[2] A. Borgstrom and K. Ohlsson, *Scand. J. Clin. Lab. Invest.* 36, 809 (1976).
[3] C. Largman, J. W. Brodrick, M. C. Geokas, and J. H. Johnson, *Biochim. Biophys. Acta* 543, 450 (1978).

ISBN 0-12-181974-4

elastase 2,[4-6] and chymotrypsin.[7] The ability to quantitate the levels of total immunoreactive trypsin in biological fluids as well as to identify the molecular forms of trypsin present in the sample has resulted in several reports of clinical applications for the radioimmunoassay of cationic trypsin. These include the diagnosis of cystic fibrosis,[8-10] the possible diagnosis of acute pancreatic inflammatory disease,[11,12] the possible differential diagnosis of pancreatic cancer from chronic pancreatic inflammation,[13-16] studies on diabetes,[17,18] and the possibility of noninvasive assessment of pancreatic function.[8,19,20]

Previous attempts at detection of pancreatic endopeptidases in plasma using enzymatic assays have been unsuccessful for two major reasons: (1) Enzymatic methods depend on catalytic activity that may be masked by plasma protease inhibitors or be absent in the case of the zymogen form. (2) Low-molecular-weight amide or ester substrates are not specific for pancreatic endopeptidases. The use of an immunologic method allows the detection of enzymatically inactive forms of the proteases, including the zymogen and proteases bound to α_1-protease inhibitor. Furthermore, the high degree of specificity inherent in the radioimmunoassay permits the identification of proteins of pancreatic origin in blood and other biological fluids. The use of a radiolabel permits the immunologic assay to detect

[4] M. C. Geokas, J. W. Brodrick, J. H. Johnson, and C. Largman, *J. Biol. Chem.* **252**, 61 (1977).
[5] C. Largman, J. W. Brodrick, M. C. Geokas, J. H. Johnson, and M. Fassett, *Am. J. Physiol.* **238**, G177 (1980).
[6] A. Borgstrom, J. Kukora, and K. Ohlsson, *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 633 (1980).
[7] M. C. Geokas, C. Largman, J. W. Brodrick, J. H. Johnson, and M. Fassett, *J. Biol. Chem.* **254**, 2775 (1979).
[8] P. R. Durie, C. Largman, J. W. Brodrick, J. H. Johnson, K. J. Gaskin, G. G. Forstner, and M. C. Geokas, *Pediatric Research*, in press.
[9] J. R. Crossley, R. B. Elliot, and P. A. Smith, *Lancet* **1**, 472 (1979).
[10] D. N. King, A. F. Heeley, M. P. Walsh, and J. A. Kuzemko, *Lancet* **2**, 1217 (1979).
[11] J. W. Brodrick, M. C. Geokas, C. Largman, M. Fassett, and J. H. Johnson, *Am. J. Physiol.* **237**, E474 (1979).
[12] E. Elias, M. Redshaw, and T. Wood, *Lancet* **2**, 66 (1977).
[13] A. Borgstrom and K. Ohlsson, *Eur. J. Clin. Invest.* **8**, 379 (1978).
[14] G. Lake-Bakaar, S. McKavanagh, and J. A. Summerfield, *Lancet* **2**, 878 (1979).
[15] T. E. Adrian, H. S. Besterman, C. N. Mallinson, A. Pera, M. R. Redshaw, T. P. Wood, and S. R. Bloom, *Clin. Chim. Acta* **97**, 205 (1979).
[16] H. Koop, P. G. Lankisch, F. Stockman, and R. Arnold, *Digestion* **20**, 151 (1980).
[17] D. R. Gamble, A. Moffatt, and V. Marks, *J. Clin. Pathol.* **32**, 897 (1979).
[18] T. E. Adrian, A. J. Barnes, and S. R. Bloom, *Clin. Chim. Acta* **97**, 213 (1979).
[19] G. Lake-Bakaar, S. McKavanagh, M. Redshaw, T. Wood, J. A. Summerfield, and E. Elias, *J. Clin. Pathol.* **32**, 1003 (1979).
[20] M. C. Geokas, C. Largman, E. Pasaro, P. R. Durie, and M. O'Rourke, unpublished observations (1980).

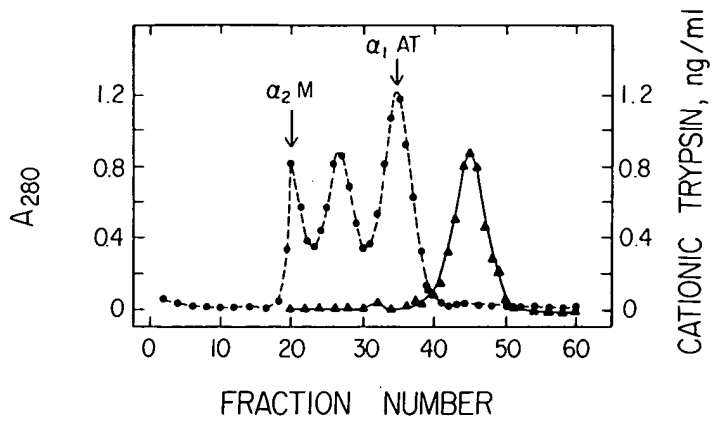
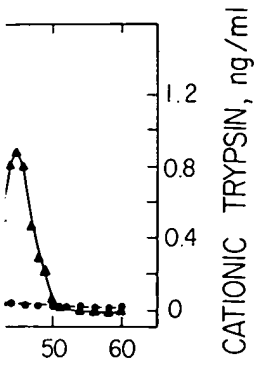


FIG. 1. Molecular size distribution of immunoreactive pancreatic cationic trypsin in pooled normal human serum. Serum was subjected to gel filtration on Sephadex G-200 in 50 m*M* Tris-HCl, 0.14 *M* NaCl (pH 7.6). Aliquots of 200 μl of fractions indicated were assayed in duplicate. Immunoreactive cationic trypsin in fractions other than those shown was below minimum detectable dose. ▲——▲, Immunoreactive cationic trypsin; ●---●, A_{280}. Immunoreactive material in fractions 40–50 was shown to be trypsinogen by activation with enteropeptidase followed by affinity chromatography on a column of lima bean trypsin inhibitor bound to Sepharose. α_2M, α_2-Macroglobulin; α_1AT, α_1-protease inhibitor.

circulating forms of the pancreatic endopeptidases that are present in normal blood in the 5–25 ng range.

The radioimmunoassay methodology has been employed in conjunction with molecular size fractionation of plasma or serum on Sephadex G-200 or BioGel A-0.5 *M* columns to demonstrate that cationic[1,2] and anionic[3] trypsinogen are the only immunoreactive forms of the respective trypsins present in plasma or serum of normal individuals. A typical gel filtration experiment for the characterization of immunoreactive cationic trypsin in normal serum is shown in Fig. 1. Furthermore, trypsinogen appears to be the only immunoreactive form of trypsin present in plasma samples obtained from infants with cystic fibrosis, in which significantly increased levels of total immunoreactive cationic trypsin are found.[8] In contrast to the trypsinogens, all of the immunoreactive elastase 2 in normal plasma is present as a complex of proelastase 2 with α_1-protease inhibitor[5] (also referred to as α_1-anti-trypsin); whereas immunoreactive human chymotrypsin is present in plasma as both the free zymogen and as immunoreactive chymotrypsin bound to α_1-protease inhibitor[7] (Fig. 2).

The distribution of immunoreactive trypsin in serum from a patient with acute pancreatic inflammatory disease is shown in Fig. 3. In addition to the zymogen, trypsin bound to two plasma protease inhibitors (α_2-



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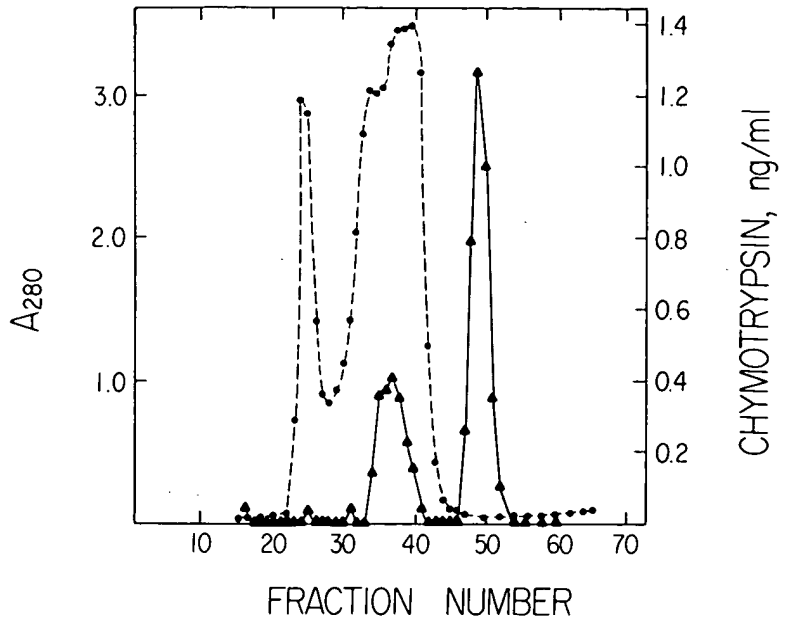


FIG. 2. Molecular size distribution of immunoreactive pancreatic chymotrypsin in human plasma. Plasma was subjected to gel filtration on BioGel A-0.5 *M* in 50 m*M* Tris-HCl, 0.14 *M* NaCl (pH 7.6). Aliquots of 200 μl were assayed in duplicate. ▲——▲, Immunoreactive chymotrypsin; ●---●, A_{280}.

macroglobulin and α_1-protease inhibitor) can be detected.[11] Investigation of patient plasma samples[11] and experiments in dogs[21] suggest that the degree of conversion of trypsinogen to α_2-macroglobulin-bound trypsin may be correlated with the severity of the disease.

The use of the radioimmunoassay technique has also facilitated the investigation of the pathway of entrance of pancreatic proenzymes into the circulation. Experiments in dogs have demonstrated that pancreatic proelastase[22] and two trypsinogens[20] enter the bloodstream directly from the pancreas rather than by absorption from the intestine. Both the factors controlling the entrance of pancreatic zymogens into the bloodstream and the relationship between the amount of immunoreactive trypsinogen entering the circulation and that present in pancreatic secretion have been studied using radioimmunoassay.[20] The mechanism of clearance of cir-

[21] M. C. Geokas, C. Largman, P. R. Durie, J. W. Brodrick, S. B. Ray, and M. O'Rourke, unpublished observations (1980).

[22] M. C. Geokas, C. Largman, J. W. Brodrick, and M. Fassett, *Am. J. Physiol.* 238, G238 (1980).

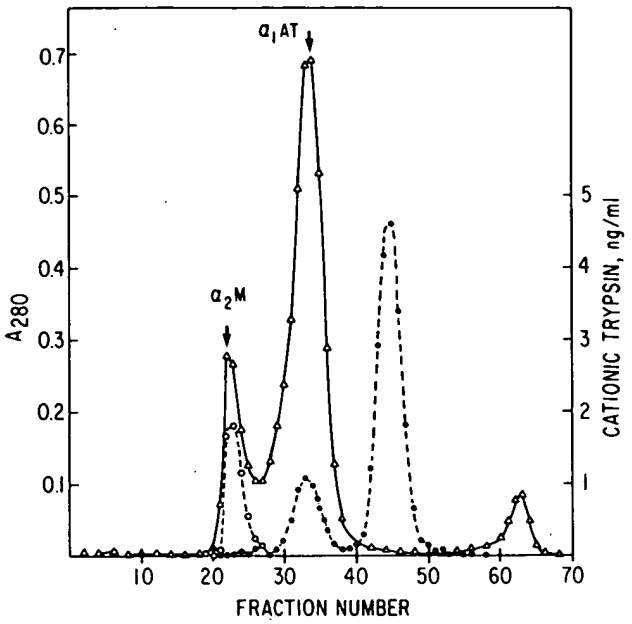


FIG. 3. Molecular size distribution of immunoreactive pancreatic trypsin in serum from a patient with acute pancreatitis. An aliquot (200 μl) of patient serum was subjected to gel filtration on BioGel A-0.5 *M* in 50 m*M* Tris-HCl, 0.14 *M* NaCl (pH 7.6). Aliquots of 200 μl were assayed for immunoreactive cationic trypsin. To detect α_2-macroglobulin-bound cationic trypsin, aliquots of 200 μl of the fractions containing α_2-macroglobulin were adjusted to pH 3 with 2 *M* formic acid, incubated for 1 hr at 37°, and assayed for immunoreactive trypsin. ●---●, Immunoreactive cationic trypsin in samples without incubation at pH 3; ○---○, immunoreactive cationic trypsin after incubation at pH 3; △——△, A_{280}.

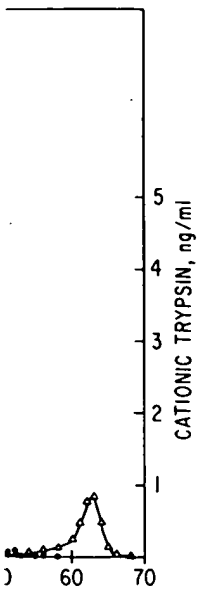
culating pancreatic zymogens in the dog[23] and in man[13,20] has also been investigated using radioimmunoassay. These studies have demonstrated that the kidney is a major site of clearance of pancreatic zymogens from the bloodstream.

The numerous potential applications of the radioimmunoassay technique for pancreatic proteases have resulted in the production of two commercial radioimmunoassay procedures for human trypsin in kit form.[24,25] While these assays are not described in detail for proprietary reasons, it appears that neither assay satisfactorily solves the potential problems that concern the binding of labeled antigen tracer to plasma inhibitors and that are described in the following sections.

[23] M. C. Geokas, C. Largman, P. R. Durie, E. Pasaro, and M. O'Rourke, unpublished observations (1980).

[24] RIA-gnost Trypsin, Behringwerke, Marburg/Lahn, Federal Republic of Germany.

[25] CIS Trypsin radioimmunoassay kit, Eurotope Services Ltd, Sorin Biomedica-Gruppo Radiochimica, Italy.



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Radioimmunoassay Procedure

The radioimmunoassay consists of three steps: (1) A competition step in which labeled tracer and either standard antigen or sample antigen compete for limiting specific antisera. (2) A separation step in which antibody-bound label is separated from free label. (3) The data collection and calculation process. The development of a successful radioimmunoassay technique requires purified enzyme for use as standard, in preparation of radiolabeled tracer, and for the production of high-titer antisera. Several additional factors must be determined for the assay, including conditions of incubation, optimal method for separation of bound from free tracer, and the method of data analysis. Each of these ingredients and factors are described in detail in the following sections.

Preparation of Antigen for Immunization and Standard

Because the radioimmunoassay is designed to measure the concentration of unspecified proteins that compete with a labeled tracer for antibody, the fidelity of the assay is primarily dependent on the purity of the material employed as tracer. It is not essential, however, for the antigen used as standard or for immunization to be pure, if the concentration of the antigen to be measured is known in the material employed as standard. Although impure preparations of standard have occasionally been used[12,26] the necessity of obtaining homogeneous material for preparation of tracer has resulted in the use of homogeneous antigen as standard by most investigators.[1-4,7,22,23,27-29]

The choice of the chemical form of the antigen to be employed as standard is somewhat arbitrary because, as described later, the labeled tracer is an active site-inhibited derivative of the enzyme and the material measured in plasma is present as either the zymogen or plasma inhibitor-bound forms of the enzyme. Thus the material utilized as standard will not be identical to material being detected in plasma. Although most assays have employed the active enzyme as standard,[1,3,4,7,22,23,27,29] diisopropylfluorophosphate inactivated trypsin[2] and chymotrypsinogen[28] have also been used. If a homogeneous preparation of antigen is employed as standard, the concentration can be determined directly from the absorbance at 280 nm if the extinction coefficient has been determined. A few assays have relied on the activity of the enzyme preparation as a measure of

[26] T. P. Wood, M. R. Redshaw, and R. H. Rousell, *Lancet* 1, 781 (1979).
[27] R. S. Temler and J.-P. Felber, *Biochim. Biophys. Acta* 236, 78 (1971).
[28] R. S. Temler and J.-P. Felber, *Biochim. Biophys. Acta* 445, 720 (1976).
[29] C. Largman, M. C. Geokas, and J. W. Brodrick, unpublished observations (1980).

concentration.[12,26,30] This procedure requires knowledge of the specific activity of the homogeneous enzyme.[26] In addition, the absence of a significant amount of inactive enzyme in the preparation must be demonstrated.

Preparation and Storage of Pancreatic Cationic Trypsin Standard. The absorbance at 280 nm of a solution of homogeneous cationic trypsin is accurately determined. The concentration of cationic trypsin is calculated from the extinction coefficient of $E^{1\%}_{280} = 14.8$.[31] Following determination of the absorbance, the solution is adjusted to 0.1% crystalline bovine serum albumin (BSA) by addition of a 10% BSA solution. The standard is divided into 100-μl aliquots and stored in glass vials at $-76°$. Normally standards are used once and refrozen. When all vials have been used once, vials are used a second time if the standard curve remains unchanged. Although reuse of standard has proven satisfactory for certain assays,[1,22] this practice has been observed to lead to a change in the slope of the standard curve in the radioimmunoassay of elastase 2.[4]

Production of Antisera

Antibodies have been produced against the major pancreatic endopeptidases from several species. A partial list of antisera produced include antibodies against human anionic[3] and cationic[1,2,12,28,32] trypsin, chymotrypsin,[7,32] and elastase 2[4,33]; bovine trypsin,[34,35] chymotrypsin,[27,34,36] and chymotrypsinogen[27]; canine cationic and anionic trypsin,[37] cationic and anionic trypsinogen,[29] and elastase[22,30]; porcine trypsin and elastase[38]; and rat trypsin,[29,39] chymotrypsin,[39] and elastase.[39] In addition antisera have been produced against pancreatic exopeptidases including human[40] and porcine carboxypeptidase B[41] and porcine carboxypeptidase A.[41,42] Although most investigators have produced antisera in rabbits, other ani-

[30] J. Carballo, K. Kasahara, H. E. Appert, and J. M. Howard, *Proc. Soc. Exp. Biol. Med.* 146, 997 (1974).
[31] J. W. Brodrick, C. Largman, J. H. Johnson, and M. C. Geokas, *J. Biol. Chem.* 253, 2732 (1978).
[32] M. H. Coan, R. C. Roberts, and J. Travis, *Biochemistry* 10, 2711 (1971).
[33] K. Ohlsson and A.-S. Olsson, *Hoppe-Seyler's Z. Physiol. Chem.* 357, 1153 (1976).
[34] H. Rinderknecht, C. Carmack, and M. C. Geokas, *Immunochemistry* 12, 1 (1975).
[35] R. Arnon and B. Schechter, *Immunochemistry* 3, 451 (1966).
[36] H. G. Gundlach, *Hoppe-Seyler's Z. Physiol. Chem.* 351, 690 (1970).
[37] K. Ohlsson and H. Tegner, *Biochim. Biophys. Acta* 317, 328 (1973).
[38] B. C. McIvor and H. D. Moon, *J. Immunol.* 82, 328 (1959).
[39] S. Genell, B. E. Gustafsson, and K. Ohlsson, *Scand. J. Gastroenterol.* 12, 811 (1977).
[40] M. C. Geokas, F. Wollesen, and H. Rinderknecht, *J. Lab. Clin. Med.* 84, 574 (1974).
[41] J. T. Barrett, *Int. Arch. Allergy Appl. Immunol.* 26, 158 (1965).
[42] J. T. Barrett, *Immunology* 8, 129 (1965).

mals have occasionally been employed, including lambs[2] and guinea pigs.[27,28]

Because the pancreatic proteases possess molecular weights in the range of 23,000–30,000, these proteins generally produce very high-titer antisera. Essentially all reports of production of antisera to pancreatic proteases describe the use of an emulsion of the antigen with Freund's complete adjuvant. Early studies reported production of usable antisera with short injection schedules that generally included a series of intramuscular or intradermal injections of relatively large amounts of enzyme (10–30 mg) at 7–10 day intervals and bleedings approximately 10 days following the last injection.[35,42] Later investigators employed lower amounts of enzyme (0.2–2 mg) and added a booster injection at 2–4 weeks after the initial injection series.[30,32,41] Although antisera from this type of immunization schedule were employed for radioimmunoassay,[30] the sensitivity of the resulting assay was quite low. In order to produce high-titer antisera suitable for highly sensitive radioimmunoassays, a longer immunization schedule has been adopted. Some investigators have given repeated injections of enzyme (0.2–5 mg) at biweekly intervals for up to 6 months.[27,28,34,40,43] Recently, investigators have conserved valuable antigen by giving an abbreviated set of one to three initial injections (0.2–2 mg) followed by a widely spaced set of booster injections (0.2–2 mg).[1,3,4,7,22,23,33] Animals are bled 10 and 14 days following booster injections (10–20 ml per bleeding) and the antisera are tested to determine if suitable antibody titer for radioimmunoassay has been obtained. Antisera are tested by determining the concentration of antibody necessary to bind 50% of a standard aliquot of radioiodinated antigen prepared as described later. Figure 4 shows typical titration curves for antisera to human cationic trypsin. The antibody-bound label is separated from unbound label by a second antibody procedure. Normally, high-titer antisera are obtained by the first or second boost.

Production of Antisera to Human Cationic Trypsin. A homogeneous preparation of cationic trypsin in 1 m*M* HCl was divided into eight aliquots each containing 1 mg in 1 ml and stored at −76° until used. Individual aliquots were thawed and emulsified with 1 ml of Freund's complete adjuvant. A series of three injections were given subcutaneously at biweekly intervals, followed by intramuscular booster injections of the same antigen preparation at 3-month intervals following the third subcutaneous injection. In the case of cationic trypsin, the first set of bleedings yielded antibodies that were employed in the radioimmunoassay at a dilution of $1:1.1 \times 10^6$.

[43] R. S. Temler and J.-P. Felber, *Horm. Metab. Res., Suppl. Ser.* 5, 17 (1974).

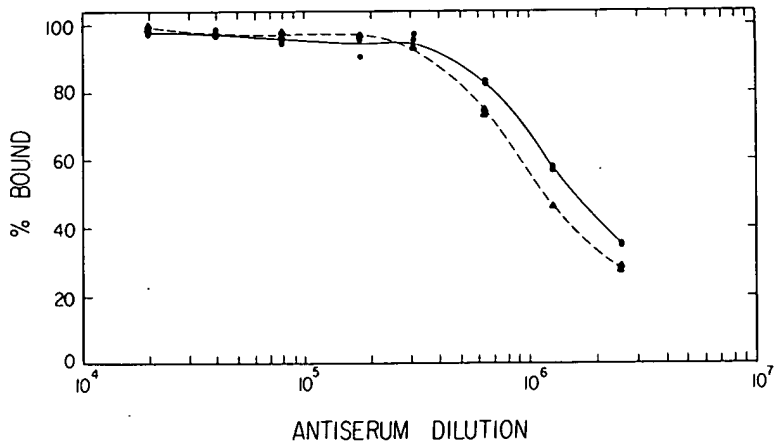


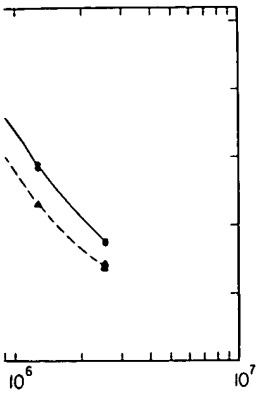
FIG. 4. Antiserum dilution curve for human pancreatic cationic trypsin. Aliquots containing 100 μl of appropriately diluted specific antibody are incubated with approximately 10,000 cpm of ^{125}I-labeled TLCK–cationic trypsin in radioimmunoassay buffer under the normal assay conditions. ▲---▲, First bleeding; ●——●, second bleeding.

Preparation of Radioiodinated Tracer

The radiolabeled antigen is the most important component of the radioimmunoassay. Because the radioimmunoassay is based on the determination of antibody-bound versus unbound labeled antigen, it is crucial that the antigen employed as labeled tracer be pure. In addition, the tracer should not bind to other proteins present in the sample incubation mixture. The fact that the pancreatic proteases bind to the plasma inhibitors α_2-macroglobulin and α_1-protease inhibitor[44] presents potential problems in the design of an appropriate tracer for the assay of an endopeptidase in a plasma sample.

Ohlsson has demonstrated the binding of trypsin to α_2-macroglobulin and α_1-protease inhibitor present in canine plasma.[45] An experiment similar to that originally performed by Ohlsson is shown in Fig. 5A, in which ^{125}I-labeled human cationic trypsin is added to human plasma and the resulting mixture is subjected to gel filtration on Sephadex G-200. As shown in Fig. 5A, 85% of the labeled active trypsin is bound to α_2-macroglobulin, while approximately 15% is bound to α_1-protease inhibitor, and a small amount of labeled material, presumably trypsin inactivated during the labeling procedure, elutes in the position of free trypsin.[1] The partition between α_2-macroglobulin and α_1-protease inhibitor varies for each protease.[1,3,4,7] However, for each human pancreatic protease examined, a substantial fraction of active enzyme added to plasma binds

[44] K. Ohlsson, *Scand. J. Clin. Lab. Invest.* 28, 5 (1971).
[45] K. Ohlsson, P.-O. Ganrot, and C.-B. Laurell, *Acta Chir. Scand.* 137, 113 (1971).



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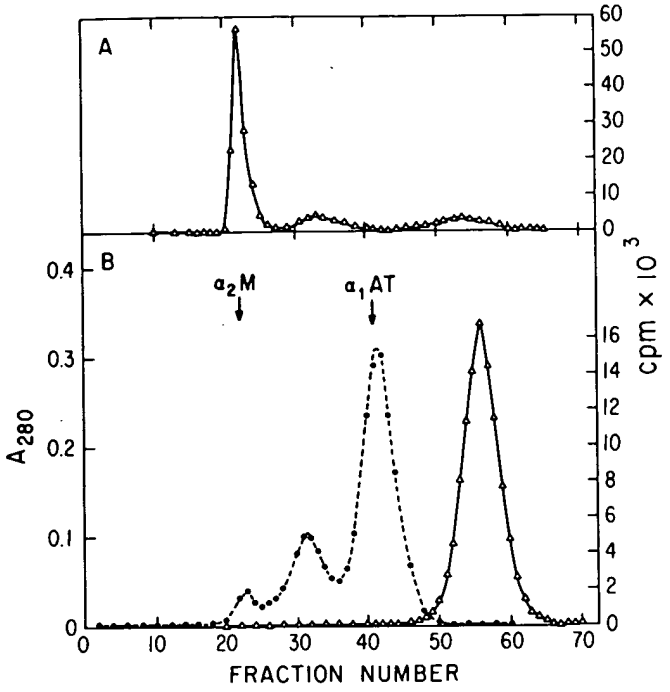

FIG. 5. Sephadex G-200 gel filtration of mixtures of ^{125}I-labeled cationic trypsin and ^{125}I-labeled TLCK–cationic trypsin with pooled normal serum. (A) ^{125}I-Labeled trypsin plus serum; (B) ^{125}I-labeled TLCK–cationic trypsin plus serum. △——△, cpm; ●---●, A_{280}, serum protein.

to α_2-macroglobulin. Because α_2-macroglobulin-bound proteases do not bind to protease specific antisera,[1,4,46] a significant percentage of the tracer is not available for antibody binding in samples in which α_2-macroglobulin is present if an active enzyme is employed as labeled tracer. To prevent tracer binding to plasma inhibitors, phenylmethane sulfonyl fluoride was used to block the active site of elastase 2 in the radioimmunoassay for this protease in human plasma.[4] This approach was based on the demonstration that enzymatic activity was a prerequisite for binding to α_2-macroglobulin[46] and α_1-protease inhibitor.[47] At approximately the same time, the use of diisopropylfluorophosphate-blocked human cationic trypsin as tracer in a radioimmunoassay for this endopeptidase in plasma was reported.[2] Tosyl-L-lysine chloromethyl ketone (TLCK) has also been used to block the active site of human cationic trypsin employed as tracer for radioimmunoassay.[1] As shown in Fig. 5B, the labeled TLCK–trypsin tracer does not bind to plasma inhibitors. This approach has been success-

[46] A. J. Barrett and P. M. Starkey, *Biochem. J.* 133, 709 (1973).
[47] A. B. Cohen, *J. Biol. Chem.* 248, 7055 (1973).

fully applied to the preparation of tracers for radioimmunoassay determination of circulating human anionic trypsin,[3] human chymotrypsin,[7] canine anionic and cationic trypsin,[23] and rat cationic trypsin.[29] The use of TLCK[48] and phenylmethane sulfonyl fluoride[49] as active site-directed inhibitors has been described in previous volumes of this series.

The use of active site-blocked enzymes for labeled tracer in the various radioimmunoassay procedures was based on the report that proteolytic activity was a prerequisite for binding to the plasma inhibitor α_2-macroglobulin.[46] This observation was based on the finding that α_2-macroglobulin was cleaved during reaction with a protease,[50] and that TLCK–trypsin did not bind to the inhibitor.[46] However, more recent studies have demonstrated that anhydrotrypsin,[51] phenazine methosulfate (PMS)-subtilisin,[51] and human proelastase 2[52] bind to α_2-macroglobulin. The importance of these findings for the radioimmunoassay of elastases was confirmed by the demonstration that the previously reported peak of immunoreactive elastase 2 associated with α_2-macroglobulin was due to labeled PMS-elastase 2 binding to the inhibitor.[5] This interference of α_2-macroglobulin in the radioimmunoassay of human elastase 2 accounted for approximately 60% of the apparent concentration of elastase 2 in plasma. Smaller peaks of apparent immunoreactive elastase, probably also due to binding of tracer by α-macroglobulins have been observed in radioimmunoassays for canine[22] and porcine[29] elastase, but no other protease radioimmunoassay has been affected by α_2-macroglobulin interference. An acid treatment step has been described that denatures α_2-macroglobulin in plasma and reduces the interference of the inhibitor in the radioimmunoassay for human elastase 2.[5] More recently, the use of a tetrapeptide chloromethyl ketone, succinyl-L-alanyl-L-alanyl-L-prolyl-L-leucine-CH_2Cl,[53] to block elastase 2 with a larger molecule designed to disrupt the tracer–α_2-macroglobulin interaction, has reduced the interference of α_2-macroglobulin in the radioimmunoassay by approximately 80%.[29]

It should be noted that several investigators have reported the use of active enzyme as tracer in radioimmunoassays of pancreatic proteases. Temler and Felber described the measurement of bovine trypsin and

[48] E. Shaw, this series, Vol. 11 [80].
[49] A. M. Gold, this series, Vol. 11 [83].
[50] P. C. Harpel, *J. Exp. Med.* 138, 508 (1973).
[51] D. Tsueu, K. Kado, K. Fujiwara, M. Tomimatsu, and K. Ogita, *J. Biochem.* (*Tokyo*) 83, 1345 (1978).
[52] C. Largman, J. W. Brodrick, M. C. Geokas, W. M. Sischo, and J. H. Johnson, *J. Biol. Chem.* 254, 8516 (1979).
[53] C. Largman, E. G. Delmar, J. W. Brodrick, M. Fassett, and M. C. Geokas, *Biochim. Biophys. Acta* 614, 113 (1980).
[54] W. M. Hunter and F. C. Greenwood, *Nature* (*London*) 194, 495 (1962).

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h a larger molecule designed to
raction, has reduced the inter-
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)rement of bovine trypsin and

, and K. Ogita, *J. Biochem.* (*Tokyo*) 83,

M. Sischo, and J. H. Johnson, *J. Biol.*

Fassett, and M. C. Geokas, *Biochim.*

)ndon) 194, 495 (1962).

chymotrypsin and their respective zymogens in pancreatic juice by radioimmunoassay.[27,43] In these assays, active enzyme was successfully used as labeled tracer because no plasma samples were studied. However, these authors also reported determination of human trypsin in plasma using a radioimmunoassay employing labeled active trypsin as tracer.[28] In this assay procedure, bovine plasma was added to all assay tubes. It appears likely that a portion of labeled trypsin reacted with bovine plasma α_1-protease inhibitor and the resulting complex acted as a blocked form of tracer for the assay of human plasma samples.

In a similar manner, a radioimmunoassay for human pancreatic trypsin in plasma was described in which it appeared that active trypsin is employed as tracer.[12] However, subsequent publications describing the same assay note that the trypsin used as tracer is reacted with an unspecified protease inhibitor to prevent autolysis,[15,26] a process that would form a protease–inhibitor complex that might be a viable form of tracer for assay of plasma fractions. Because this assay[12] forms the basis of a commercially available kit for determination of human trypsin in plasma,[24] it is important to realize that this technique yields from 6[15] to 16[26] times higher levels of pancreatic cationic immunoreactive trypsin in human plasma than the mean value of 25 ng/ml detected by assays using TLCK–trypsin[1] or DIP–trypsin[2] as labeled tracer. This assay[12] has been criticized for yielding an artifactual peak of immunoreactive trypsin in fractionated plasma, which was suggested to result from binding of tracer to α_2-macroglobulin.[53]

Most of the proteins employed as tracer are iodinated using a modification of the chloramine-T procedure developed by Hunter and Greenwood[54] as described later for iodination of TLCK–cationic trypsin. Because this procedure depends on the presence of an exposed tyrosine residue, there may be instances in which the chloramine-T procedure does not yield an acceptable incorporation of ^{125}I. In these situations, an indirect labeling procedure described by Bolton and Hunter[55] is employed in which 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester is prelabeled with ^{125}I and the labeled reagent is then reacted with free amino groups in the protein. This procedure has been employed for the preparation of [^{125}I]PMS–chymotrypsin II.[7]

Preparation of ^{125}I-Labeled TLCK–Cationic Trypsin[1]

Approximately 5 μg of TLCK–cationic trypsin in 10–15 ml of 1 m*M* HCl is placed in a 12 × 75-mm disposable glass tube at room temperature. The following reagents are added as rapidly as possible: (1) 10 μl of 0.25 *M* sodium phosphate (pH 7.5) containing 0.5 mCi ^{125}I as NaI; (2) 10 μl of 4.8

[55] A. E. Bolton and W. M. Hunter, *Biochem. J.* 133, 529 (1973).

m*M* chloramine-T in phosphate buffer. The reaction tube is gently and briefly mixed by hand vortexing, and the reaction is terminated after 45 sec by addition of 10 μl of 13 m*M* sodium metabisulfite. Ten microliters of a 3% solution of fraction V BSA, followed by 50 μl of 10 m*M* Tris-HCl, 0.14 *M* NaCl (pH 7.6) are then added. The resulting mixture is applied to a 1.2 × 20-cm column of Sephadex G-75 equilibrated in 10 m*M* Tris-HCl, 0.14 *M* NaCl (pH 7.6). Prior to each iodination, the column is washed with approximately 1 ml of 3% BSA to remove adsorbed proteins from previous iodinations as well as to coat potential protein binding sites in the column. The iodination reaction tube is washed with 100 μl of the column buffer to ensure transfer of the labeled protein to the column. Fractions of 0.8 ml are collected into disposable glass tubes containing 30 μl of 3% (w/v) BSA. Radioactivity in the fractions is monitored using a Searle Gamma Survey Meter. Fractions corresponding to the elution volume of TLCK–cationic trypsin are employed as tracer in the radioimmunoassay. Usually about 80% of the radioactivity applied is protein bound. The specific activity of the tracer is approximately 60–80 μCi/μg. Peak fractions are stored at −20° for later use after repurification by the same Sephadex G-75 procedure. Under these conditions the tracer is stable for approximately 1 month. Longer storage times have not been used to date.

Equilibration Conditions

Although many radioimmunoassay procedures for hormones employ a nonequilibrium competition process, most of the radioimmunoassays described for pancreatic enzymes allow the incubation mixture of tracer, antigen, and antibody to approach equilibrium in order to increase the sensitivity of the assay. Experiments using radioimmunoassays for human carboxypeptidase B[40] and elastase 2[4] demonstrated that when the equilibration step was carried out at 4°, sensitivity increased with successive days of incubation up to 4 days. However, 5 or 6 days of incubation did not lead to significant increases in sensitivity. An equilibration step of 4 days at 4° has been employed for most assays,[1,3,4,7,22,29] although an equilibration step of 24 hr at 15° also has been used without substantial loss of sensitivity.[22,23] Other assays have employed equilibration steps at 4° for 24 hr[2,12] and 3 days.[28] A nonequilibrium incubation for 1 hr at room temperature has been described.[25]

Separation of Bound and Free Tracer

The separation of antibody-bound tracer from free tracer is usually accomplished using the second antibody technique.[36] This procedure in-

[36] W. D. Odell, A. F. Parlow, C. M. Cargille, and G. T. Ross, *J. Clin. Invest.* 47, 2551 (1968).

e reaction tube is gently and
eaction is terminated after 45
etabisulfite. Ten microliters of
by 50 μl of 10 m*M* Tris-HCl,
sulting mixture is applied to a
ilibrated in 10 m*M* Tris-HCl,
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r from free tracer is usually
hnique.[56] This procedure in-

oss, *J. Clin. Invest.* 47, 2551 (1968).

volves the addition of a solution of second antibody directed against the IgG of the species in which the specific antisera was raised in order to form an insoluble antibody–antigen matrix. Second antibody incubations at 4° for 24 hr[1,3,4,7,22,29] or 6 hr[2,12] have been reported, and an incubation at 15° for 6 hr has also been employed.[22,23] Following the second incubation, the tubes are centrifuged to pellet the second antibody-specific IgG complex that contains a fraction of the labeled tracer. The normal procedure is to discard the supernatant and count the pellet, although counting the supernatant can also be utilized. The use of a second antibody technique necessitates the addition of normal carrier IgG to the equilibration incubation in order to raise the concentration of IgG sufficiently for efficient pellet formation upon addition of the second antibody. We have repeatedly observed that different preparations of normal rabbit IgG change the dilution of specific antiserum necessary to bind 50% of the labeled tracer. Furthermore, the effect of normal IgG does not influence the various radioimmunoassays to the same extent. This finding necessitates an antiserum titration for each assay when a new batch of IgG is employed. To eliminate the time-consuming process of preparing normal IgG and large amounts of expensive second antibody IgG, as well as the necessity of performing titration curves for each new preparation of normal IgG, the use of second antibody bound to agarose beads has been explored.[57] The advantage of the solid phase second antibody is that addition of normal IgG to the equilibration incubation mixture is unnecessary. Following a second incubation period, the beads are pelleted by centrifugation in a manner similar to the antigen–antibody complex described earlier. The agarose beads are reported to be compatible with at least one brand of automatic pipetting device.[58]

Preparation of Normal Carrier IgG. Normal rabbit IgG is prepared from rabbit serum by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography. To 200 ml of normal rabbit serum are added 133 ml of saturated $(NH_4)_2SO_4$ solution, for a final concentration of 40% of saturation. After 1 hr at 4°, the precipitate is collected by centrifugation for 10 min at 10,000 rpm. The precipitate is suspended by trituration in 100 ml of 40%-saturated $(NH_4)_2SO_4$ in water followed by centrifugation as previously described. The precipitate is then dissolved in a minimal volume of water, dialyzed for 1 hr versus water, and against two changes of 17.5 m*M* sodium phosphate (pH 6.3), and passed through a column (4 × 40 cm) of DEAE-cellulose (Whatman, DE23) equilibrated with the same buffer. Void volume fractions are combined, concentrated by ultrafiltration to an A_{280} of 18 [1.2% (w/v)], and stored at −20°.

[57] Immunobead reagent, Bio-Rad Laboratories, Richmond, California.
[58] Micromedic Systems, Hayward, California, personal communication (1980).

Preparation of Second Antibody IgG. γ-Globulin fraction is prepared from goat anti-rabbit γ-globulin antiserum by the aforementioned procedure, except that DEAE-cellulose chromatography is not performed. After dialysis the dissolved $(NH_4)_2SO_4$ pellet is centrifuged to remove insoluble protein and diluted to one-half of the original volume of antiserum used. To this solution are added an equal volume of water and two volumes of 50 m*M* Tris-HCl, 0.02 *M* Na_2EDTA (pH 7.6). The resulting solution is stored at −20° prior to use in the radioimmunoassay.

Use of Solid-Phase Second Antibody. If the Immunobead procedure is employed, normal rabbit IgG is omitted from the radioimmunoassay buffer. After the first incubation period, the Immunobead reagent is added in place of second antibody IgG as follows: Immunobead reagent (200 mg) is reconstituted with 40 ml of 50 m*M* Tris-HCl, 0.14 *M* NaCl (pH 7.6). The Immunobead solution is recycled through the automatic pipetting apparatus several times immediately prior to addition to samples. A 200-μl aliquot is added to each sample. Samples are incubated for 5 hr at 15° or 24 hr at 4°, centrifuged, and counted as previously described.

Radioimmunoassay Conditions for Human Cationic Trypsin

All reagents are diluted with standard radioimmunoassay buffer containing 50 m*M* Tris-HCl buffer (pH 7.6), 0.14 *M* NaCl, 0.4% (w/v) BSA, and 0.12% normal rabbit IgG (the latter employed to assure consistent precipitation by the double antibody method). After addition of standard buffer to make a final volume of 1.0 ml, the following reagents are added, in the order indicated: 0 to 150 μl of standard cationic trypsin or serum sample, 0.1 ml of cationic trypsin antiserum diluted 1 : 110,000 with standard buffer, and 0.1 ml of ^{125}I-labeled TLCK–cationic trypsin (10,000 cpm). After incubation for 4 days at 4°, bound and free labeled antigen are separated by the double antibody procedure. An aliquot of 0.1 ml of γ-globulin fraction from goat anti-rabbit γ-globulin antiserum is added to each assay tube, followed by incubation for 24 hr at 4°. The average total counts per minute per tube is determined by counting ten tubes, and the bound fraction in each assay tube is then isolated by centrifugation for 10 min at 6000 rpm in the Sorvall HS-4 rotor. After careful aspiration of the supernatants, radioactivity in the pellets is quantitated with a Searle Model 1185 γ scintillation system. All values are corrected for nonspecific counts per minute measured in the absence of anticationic trypsin antiserum.

Radioimmunoassay Data Analysis

Many methods have been devised for treatment of radioimmunoassay data obtained with standard curves and aliquots of unknown samples.

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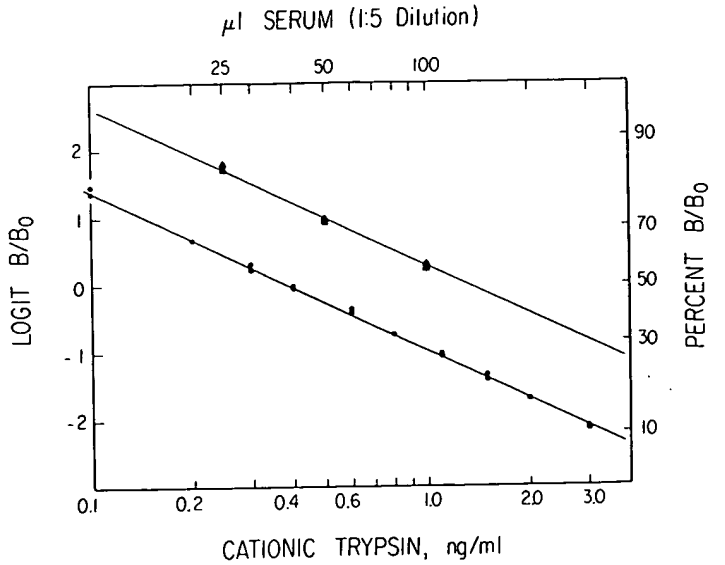

FIG. 6. Cationic trypsin standard curve and serum dilution curve. Lines were constructed from slopes and intercepts obtained by weighted linear regression analysis. ●——●, Cationic trypsin standard curve; ▲——▲, pooled normal serum diluted 1 : 5 with standard radioimmunoassay buffer.

They vary in complexity from simply plotting standard curve data and interpolating unknown values graphically to sophisticated BASIC or FORTRAN IV computer analysis of the variance at different levels of a linearized standard curve to allow for accurate estimates of the confidence of an unknown value. A book edited by Odell and Daughaday[59] contains one of the more complete theoretical as well as practical discussions, from several viewpoints, of the types of data analysis that have been used.

In our laboratory the FORTRAN IV data analysis programs developed by Rodbard and co-workers[60–63] are employed. Paper tape output from the γ counter is used as input data to a time-shared Digital Equipment Co. Lab. 8/E minicomputer. The programs of Rodbard and Frazier were modified as necessary in terms of input and output, but the mathematical

[59] W. D. Odell and W. H. Daughaday, eds., "Principles of Competitive Protein-Binding Assays." Lippincott, Philadelphia, Pennsylvania, 1972.
[60] D. Rodbard, P. L. Rayford, J. A. Cooper, and G. T. Ross, *J. Clin. Endocrinol. Metab.* 28, 1412 (1968).
[61] D. Rodbard and J. F. Lewald, *Acta Endocrinol. (Copenhagen)* 64, Suppl. 147, 79 (1970).
[62] D. Rodbard, *Adv. Exp. Med. Biol.* 36, 289 (1973).
[63] D. Rodbard and G. R. Frazier, "Radioimmunoassay Data Processing," 2nd ed., PB 217366, PB 217367. U.S. Dept. of Commerce, Natl. Tech. Inf. Serv., Springfield, Virginia, 1972.

treatment was left intact. The method consists of linearizing the standard curve data by use of the logit transformation and of using an iterative, weighted least squares regression analysis of logit(B/B_0) versus $\log_e$ of standard dose, where B is net bound counts per minute for an experimental point, B_0 is net bound counts per minute for zero dose, and logit(B/B_0) is $\log_e [(B/B_0)/(1 - B/B_0)]$. Sample values are calculated from the slope and intercept of the standard curve. Generally three levels of sample are assayed in duplicate over a fourfold concentration range. Figure 6 shows a standard dose–response curve for cationic trypsin plotted according to this method. Also shown are the experimental points obtained for a pool of five normal sera. The slope of the serum dilution curve obtained by the weighted linear regression is parallel to the standard curve by a Student's t test for slopes, indicating that concentrations calculated from different assay levels are equally valid. Unknown values are considered valid if the standard deviation is less than 10% of the mean and all points fall within the range $0.85 > B/B_0 > 0.15$. We use this method because it is one of the more powerful types of data analysis when a large enough computer is available. We prefer a plot of B/B_0 versus log dose when graphical methods are to be employed. The standard curve obtained should have a linear region between $0.8 \geq B/B_0 \geq 0.2$. Thus, if unknown values are interpolated only if they fall in this range, maximum confidence in their accuracy can be expected.

A selection of assay statistics for six consecutive experiments is shown in Table I. The minimum detectable dose is that required to lower the bound counts per minute by two standard deviations from B_0, based upon the calculated variance in the standard curve at zero dose. Based on the mean of the minimal detectable dose levels shown, we estimate that 0.6 ng/ml of cationic trypsin can be detected in a 100-μl sample. The between-assay variance obtained by eight consecutive assays of a pool of five normal sera is about 14%. In a single assay set for cationic trypsin, an average value of 26 ± 9.3 ng/ml was obtained with serum samples obtained from 15 normal healthy adult volunteers.

Specificity of the Radioimmunoassay

Despite the known sequence similarities of the pancreatic endopeptidases, the radioimmunoassays for these enzymes are, in general, remarkably specific. Table II shows the cross-reactivities of other human pancreatic endopeptidases and of trypsin–inhibitor complexes in the radioimmunoassay for human cationic trypsin. Only anionic trypsin cross-reacts to a significant extent, and its dose–response curve is not parallel to the standard curve. We have observed substantial cross-

TABLE I
ASSAY STATISTICS

Date of standard curve	Slope	Correlation coefficient	$X_{0.5}$[a] (ng)	Minimum detectable dose (ng)
11/12	−1.031	−0.999	0.346	0.008
11/24	−1.069	−0.999	0.372	0.011
12/8	−1.093	−0.999	0.367	0.013
12/10	−1.030	−0.999	0.336	0.011
12/16	−1.088	−0.998	0.319	0.016
12/17	−1.069	−0.998	0.342	0.018

[a] Dose required for $B/B_0 = 0.5$, or for $\text{logit}(B/B_0) = 0$.

reactivity between human chymotrypsins I and II; however, these proteins may be the products of a single gene. Elastase purified from baboon pancreas cross-reacts only 0.5% in the human elastase 2 radioimmunoassay, and human leukocyte elastase cross-reacts by less than 0.1%. No measurable cross-reactivity has been detected between bovine, porcine, or canine trypsin in the radioimmunoassays for either human trypsin.

Summary

The radioimmunoassay methodology has been employed to demonstrate that the zymogen forms of the pancreatic proteases are present in normal plasma, whereas in acute pancreatic inflammation both the zymo-

TABLE II
CROSS-REACTIVITIES OF PROTEASES AND CATIONIC TRYPSIN–INHIBITOR COMPLEXES

Enzyme or enzyme–inhibitor complex	Cross-reactivity (%)
Cationic trypsinogen	98
Human anionic trypsin	< 0.6–2.5 >
Human chymotrypsin I	0
Human chymotrypsin II	0.8
Human elastase 1	0.8
Human elastase 2	0
Bovine trypsin	0
Cationic trypsin–α_1-protease inhibitor	77.4
Control: α_1-protease inhibitor alone	<0.1
Cationic trypsin–α_2-macroglobulin	1.5
Control: α_2-macroglobulin alone	<0.1

gens and the α_2-macroglogulin and α_1-protease inhibitor bound forms can be detected in the bloodstream. The pancreatic endopeptidases have been shown to be excellent antigens and specific antibodies of high titer and affinity can easily be obtained. Radioiodination of these proteins is easily achieved due to the presence of the exposed tyrosine residues.

The use of active site-inhibited forms of the endopeptidases as radiolabeled tracer is necessary in order to prevent tracer binding to plasma inhibitors. In this regard, the use of the proenzyme form of the endopeptidases as labeled tracer has not been pursued because of potential activation in the assay by plasma proteases. The separation of antibody-bound tracer from free tracer is accomplished without difficulty.

The lack of cross-reactivity in the radioimmunoassay of a given pancreatic endopeptidase with the same protease from other mammalian species demonstrates that a separate radioimmunoassay must be developed for each enzyme to be investigated. Furthermore, the lack of cross-reactivity between the various pancreatic endopeptidases of the same species permits the specific identification of each protease in a biological sample. The radioimmunoassays for pancreatic endopeptidases have been useful as investigative tools as well as for diagnostic purposes. We believe that the radioimmunoassays for human pancreatic proteases will be important in the continuing exploration of the role of these enzymes in health and disease.

Acknowledgment

We would like to thank Janice H. Johnson for invaluable assistance during the development of the radioimmunoassays for pancreatic endopeptidases and for helpful discussions during the preparation of this chapter. This work was supported by the Medical Research Service of the Veterans Administration and the Council for Tobacco Research-USA, Inc.

[18] Radioimmunoassay of Human Pancreatic Amylase

By MICHIO OGAWA, YUICHI TAKATSUKA, TAKESHI KITAHARA, KISHIO MATSUURA, and GORO KOSAKI

Serum amylase determination has been the most commonly used test for the diagnosis of pancreatic inflammation.

It is known, however, that the serum amylase level falls rapidly to normal or below normal and could not be correlated with the subsequent

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